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Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Yeast strains with improved fructose fermentation capacity

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YEAST STRAINS WITH IMPROVED FRUCTOSE FERMENTATION CAPACITY

The present invention relates to yeast strains with improved fructose fermentation capacity, in particular to strains transformed with a gene encoding an improved hexose transporter gene, more in particular a mutated *HXT3* gene.

During the alcoholic fermentation of wine, hexoses such as glucose and fructose are converted into alcohol by microbial activity, in particular by yeast strains of the genus *Saccharomyces*. *S. cerevisiae* is a preferred yeast in wine-making and selected strains of *S. cerevisiae* are used as starters to inoculate grape musts and perform the alcoholic fermentation. Grape musts contain equivalent amounts of glucose and fructose whereas the level of total hexoses typically ranges from 160 to 300 g/L. *S. cerevisiae* is a glucophilic yeast, preferring glucose to any other carbon source that may be present in the growth substrate. As a result, the fructose/glucose ratio of the must progressively increases during the alcoholic fermentation. It has indeed been confirmed that in bottled wine, fructose is always found in larger amounts than glucose. During fermentation, a strong imbalance in the ratio of fructose and glucose is assumed to be a leading cause of stuck fermentation.

On a molecular level, the fermentation capacity of yeasts has been studied quite extensively. One of the early steps in the metabolism of sugars by the action of yeast is the transport of the sugars across the plasma membrane. Specific genes encoding transporters for different sugars are expressed in yeast. In *Saccharomyces*, the uptake of hexoses such as glucose and fructose, is mediated by specific hexose transporters that belong to a superfamily of monosaccharide facilitators (Reifenberger, E., Freidel, K. and Ciriacy, M. (1995) **16**(1), 157-167). To date, more than sixteen genes encoding such genes, notably the so-called *HXT*-genes (which stands for hexose transport), have been identified. The expression of individual *HXT*-genes and homologues is dependent on environmental factors, such as the hexose concentration sensed by the yeast cell. It has been proposed that the uptake of hexoses is catalysed by two kinetically different systems (Bisson, L.F., Coons, D.M., Kruckeberg, A.L. and Lewis D.A. (1993) Crit Rev Biochem Mol Biol **28**, 295-308; Lagunas, R. (1993) FEMS Microbiol Rev **104**, 229-242). One system has a high affinity for hexoses. This high affinity component is absent in cells growing in relatively high hexose concentrations, e.g. 2% glucose. Under these conditions the yeast cell

expresses low affinity transporters. Construction of mutant yeast strains lacking multiple *HXT* genes made it possible to identify the main glucose transporters in yeast (Reifenberger, E., Boles, E. and Ciriacy, M. (1997), Eur. J. Biochem. **245**: 324-333). In a yeast strain lacking the genes *HXT1* through *HXT7*, growth on media containing high and low glucose concentrations (0.1% to 5%), glucose uptake and glucose consumption were below the detection level. In a series of experiments with mutant yeast strains expressing only one of the genes *HXT1* through *HXT7*, it was shown that *HXT1*p and *HXT3*p are low-affinity transporters ($k_m \approx$ 50-100 mM hexose), *HXT4*p is moderately low, and *HXT2*p, *HXT6*p and *HXT7*p are high affinity transporters ($k_m \approx$ 1-4 mM hexose), regardless of the culture conditions of these mutants (0.1% or 5% glucose) (Reifenberger, E., Freidel, K. and Ciriacy, M. (1995) Yeast **11**, S457). All hexose carriers display a stronger affinity for glucose compared to fructose. This is especially the case for the low affinity carriers *HXT1* (110 mM for glucose versus > 300 mM for fructose) and *HXT3* (65 mM for glucose versus 125 mM for fructose).

The role of the *HXT* carriers has also been characterized during wine fermentation (Luyten, K., Riou, C. and Blondin, B. (2002) Yeast **19**, 1-15 and Riou, C. Luyten, K. de Chazal, E. and Blondin (2001) Yeast 18, S293). It was shown that under enological fermentation conditions several carriers (*HXT1, HXT3, HXT6, HXT7*) were involved in the hexose transport.

Following consumer demands and international winemaking practices, a large percentage of quality natural wines are fermented to dryness. This means that the amount of residual hexoses in the wines is usually below 1 g/L. Fructose is the main sugar present at the end of fermentations because fructose is more difficult to ferment than glucose, and therefore fermentations are often slow at the end. Depending on yeast activity, this can lead to sluggish or stuck fermentations. Yeasts that have a strong capacity to ferment fructose are expected to yield more rapid fermentation ends.

Yeast strains that are better able to ferment fructose have been isolated from nature and such so-called fructophilic yeasts have been successfully used to further reduce the sugar content of fermented must. They have also been successfully employed in stuck fermentations to eliminate the remaining sugar by inoculation with new yeast cells. A fructophilic yeast strain called Fermichamp ® has been isolated by the Institut National de Recherche Agronomique in France and made commercially available by DSM Food Specialties.

Surprisingly, it has now been found that the ability to better utilise

fructose resides in the *HXT3* gene. A detailed study has been conducted on the HXT3 gene within the genome of the Fermichamp ® strain. This particular HXT3 gene encodes a mutated hexose transporter particularly suited for the fermentation of fructose. It has also been found that transferring the mutated *HXT3* gene can transfer this ability to a non-fructophilic strain.

A number of specific mutations within the *HXT3* gene is herein identified that individually or in combination may attribute to the fructose utilizing ability of yeasts. Consequently, the invention relates to an isolated *HXT3* gene comprising one or more mutations that improve the capacity of the gene product to transport fructose. The invention also relates to the specific gene and gene products derived from Fermichamp ® as identified herein, as well as to yeast strains comprising a foreign mutated *HXT3* gene such as the Fermichamp ® gene identified herein.

In this context the term "foreign" refers to a gene that does not naturally occur in the genome of an organism, but instead has been acquired by the yeast through a recombination event, a mutation event or otherwise, such as by natural selection or by breeding.

The terms "mutated" or "mutation" or "mutations" in this context mean that a nucleotide sequence of the nucleic acid encoding the *HXT3* transporter is different in comparison to the wild type HXT3 sequence of the organism concerned. Alternatively, the terms "mutated" or "mutation" or "mutations" may refer to an alteration in the nucleotide sequence of a nucleic acid encoding the *HXT3* transporter in comparison to the sequence of the gene encoding the endogenous HXT3 transporter. Additionally, the terms "mutated" or "mutation" or "mutations" are used herein to indicate alterations in the amino acid sequence of the hexose transporters in comparison to the natural or endogenous or wild type amino acid sequence.

The mutations that attribute to the fructophilic phenotype of the Fermichamp yeast strain are Thr 200 Ala, Ile 209 Val, Met 324 Ile, Leu 388 Met, Tyr 389 Trp, Ile 392 Val, Glu 414 Gln, Gly 415 Asn, Ile 449 Val, Leu 471 Ile, wherein the first three characters indicate the amino acid of the wild type *HXT3* protein, the three digits in the middle represent the position of the mutation in the protein (Start Met = amino acid number 001) and the last three characters represent the amino acid of the mutated *HXT3* protein according to the invention.

Now that the mutations in the Fermichamp *HXT3* gene have been elucidated, it is within the routine abilities of a person skilled in the art to find equivalents that work equally well but differ somewhat in the exact number and position

of the mutations. For instance, mutants may be constructed that carry only a single mutation at at least one of the positions Thr 200, Ile 209, Met 324, Leu 388, Tyr 389, Ile 392, Glu 414, Gly 415, Ile 449, Leu 471 mentioned above. These mutants may then be tested for their ability to utilize fructose as described in the examples and advantageous recombinants may easily be selected.

Alternatively, several mutation methods, which are known in the art, may be employed to introduce mutations in the HXT3 gene of a given yeast strain in order to render the strain more fructophilic.

Also, the skilled person may find that mutations adjacent to the positions described above may yield useful recombinants. Any of the mutant *HXT3* genes mentioned above are therefore encompassed in the present invention.

Although some of the above mutations may be considered conservative substitutions, this does not mean that as such they do not contribute to the fructophilic phenotype. The most likely candidate for a substantial effect on the phenotype, however, are the non-conservative substitutions Glu 414 Gln and Gly 415 Asn.

The dominant role of these mutations in the predicted transmembrane domain 9 are in line with a possible role of this region as contact site with sugar as proposed for the related human glucose transporter Glut3 (Dwyer, D.S. (2001) Proteins **42**, 531-541). The amino acids localized in the external loop between TM9 and TM10 (Gln414, Asn415) are well positioned to interact with the substrate.

The term "conservative substitution" is intended to mean a substitution in which the wild type amino acid residue is replaced with an amino acid residue having a similar side chain. These families are known in the art and include amino acids with basic side chains (e.g.lysine, arginine and histidine), acidic side chains (e.g. aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), non-polar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). The Gly-Asn substitution is considered as a non-conservative substitution by some sources and not by others.

We show here that the *HXT3* gene plays a key role in fructose fermentation. Our data demonstrate that the special ability of Fermichamp ® to use fructose is associated with the expression of an unusual hexose transporter gene. In

particular, we have shown that the *HXT3* gene from Fermichamp ® contains 10 mutations. These mutations are responsible for the specific capacity of Fermichamp to ferment fructose since this ability can be transferred together with the mutated gene. Several mutated amino acids are located in an external loop and may contribute to this effect. It is now within the routine abilities of a person skilled in the art to determine which of these mutations is really crucial and indispensable for the altered ability of Fermichamp ® to utilize fructose.

These data allow the engineering of a number of *HXT3* carriers in order to improve the capacity to utilise fructose in any given yeast strain. More in particular, it is now possible to improve the fructose transport capacity of *HXT3* in yeast by creating mutations at or around positions Glu 414 and Gln 415 (such as Glu 414 Gln and Gly 415 Asn) or equivalent.

Analysis of the effect of overexpression of the Fermichamp *HXT3* gene shows that the mutated form of Fermichamp possesses other properties. Overexpression of this gene triggers a higher fermentation rate than a "standard" gene. This shows that the mutated protein is more efficient when over expressed, possibly because of a better folding and/or secretion and/or a higher stability.

This allows to improve the fructose utilisation of other yeasts by transfer of the *HXT3* gene from Fermichamp, as is effectively demonstrated in the examples. This can be of high interest in enology since fructose utilisation is one of the limiting factors of the fermentation rate at the end of the fermentation.

The second property that appears associated to the *HXT3* transporter, namely the capacity to enhance the fermentation-glycolytic flux when over expressed, may find also application in many fields where high fermentation rates are desirable, such as alcohol production and baking.

EXAMPLES

Example 1 Strains and culture conditions

The *S. cerevisiae* strains used in this study are listed in table 1.

Table 1. *Saccharomyces cerevisiae* strains

Strain	Genotype
Fermichamp	Industrial strain
V5	MATa *ura3 gal*
V5 HXT1-7Δ	V5 *hxt514*Δ::loxP *hxt367*Δ::loxP *hxt2*Δ::loxP
V5 HXT1-7Δ*HXT3* (V5)	V5 *hxt514*Δ::loxP *hxt367*Δ::*HXT3* from V5 *hxt2*Δ::loxP
V5 HXT1-7Δ*HXT3* (Fermichamp)	V5 *hxt514*Δ::loxP *hxt367*Δ::*HXT3* from Fermichamp *hxt2*Δ::loxP
V5 HXT1-7Δ + p*HXT3* (V5)	V5 *hxt514*Δ::loxP *hxt367*Δ::loxP *hxt2*Δ::loxP + p4H7-*HXT3*V5
V5 HXT1-7Δ + p*HXT3* (Fermichamp)	V5 *hxt514*Δ::loxP *hxt367*Δ::loxP *hxt2*Δ::loxP + p4H7-*HXT3*Fermichamp

Strain V5 is derived from the Champagne wine strain 8130. This strain was obtained by sporulation of the 8130 strain and the subsequent isolation of an ura3 mutant resistant to 5-fluoro orotic acid.

V5 HXT1-7Δ :This strain is deleted for *HXT3*-6-7 (deletion bounds localized from 1 164 600 to 1 154 055 on the chromosome IV) (positions according to *Saccharomyces cerevisiae* Genome Database, Stanford) and HXT5-1-4 (deletion bounds localized from 296 399 to 287 180 on the chromosome VIII) clusters. The strain is also deleted for HXT2 (deletion localized from position 288 125 to 289 658 of the chromosome XIII) resulting in a complete deletion of HXT1 to 7 genes. This strain is unable to grow on glucose or fructose.

Yeast strains were grown at 28°C on YPD medium (except yeast strains transformed with p4H7 plasmid) containing either 2% glucose or 2% maltose (V5 HXT1-7Δ). For assessing the growth phenotype of the different integration mutant strains, they were grown on synthetic medium (0,67% Yeast Nitrogen Base without

amino acid, 25 mg/l uracile, 5% glucose). Yeast strains transformed with p4H7plasmid containing *HXT3* transporter genes were grown on synthetic medium (see above, without uracile). Batch *enologi*cal-like fermentation experiments was carried out on synthetic must (MS300) containing 100 g/l glucose, 100 g/l fructose and an extra 115 mg/l methionine and 25 mg/l uracile (not for transformed yeast strains). This medium contains about 430 mg/l assimilable nitrogen. Precultured cells were inoculated at a density of 106 cells/ml in fermentors with a working volume of 1,1 l, equipped with fermentation locks. Fermentations were carried out at 28°C with permanent stirring (500 rpm). These conditions give fermentation kinetics similar to those of industrial scale winemaking

Example 2 Integration of *HXT3* into the V5 HXT1-7Δ strain

The *HXT3* genes originating either from V5 or Fermichamp were reintroduced into the V5 HXT1-7 deletion strain by genomic integration at the site of the original localization of their respective cluster. *HXT3* gene was amplified by PCR using primers *HXT3*P1 and I2*HXT3*. These PCR amplification products were used for genomic integration when transformed in yeast and allow integration of a single copy of a *HXT3* gene behind its own promotor. Correct integration was verified by PCR using C1*HXT3*ORF and C2*HXT3*p.primers for *HXT3*.

All the primers are listed in table 2.

Table 2. Primers used for *HXT3* integration in V5 HXT1-7

Primers	Sequence 5' to 3'	Localization
*HXT3*P1	GTGCGGGATccGAAGGCAATATC	-1128
*HXT3*P2	gatcggATCCATCATCACGTTCCTAGC	2096
I2*HXT3*	aagtgacgggcgatgagtaagaaagaaataactgactcattagaCCATCATCACGTTCCTAGC	2095
C1*HXT3*ORF	GACACAGTGACATATGCACC	168
C2*HXT3*p	TTAAGCATGATCGTCTAGGC	-1689
*HXT3*p426	aacacaaaaacaaaaagttttttaattttaatcaaaaaCTGAGTTAAACAATCATGAATTCAACTCC	-15
*HXT3*t426	*gaatgtaagcgtgacataactaattacatgactcgag*ACGGTTTAGCGTGAAATTATTTCTTGCC	1694
C1*HXT3*ORF	GACACAGTGACATATGCACC	168
C'2HXT7p426	**gccaatacttcacaatgttcg**	-125

Underlined: homology to *HXT7* terminator, UPPER CASE; homology to *HXT3*, Double underlined; homology to p4H7 promoter, *Italics*; homology to p4H7 terminator, **Bold**; homology to *HXT7* promoter.

Example 3 Construction of p4H7 multicopy plasmid containing HXT3 ORF

HXT3 genes were amplified by PCR from genomic DNA of V5 or Fermichamp strains using primers *HXT3*p426 and *HXT3*t426. The *HXT3* genes were cloned in the plasmid p4H7 described in Hamacher et al., 2002. Microbiology, vol 148, 2783-2788, by in vivo recombination in *Sacharomyces cerevisiae*. The p4H7plasmid possess a truncated HXT7 promoter and a CYC1 terminator. The p4H7plasmid was first linearized with BamH1 & EcoR1. The 5' end of primer *HXT3*p426 is homologous to the BamH1 end (HXT7 promoter) of the p4H7 plasmidlinearized with BamH1 & EcoR1. The 5' end of primer *HXT3*t426 is homologous to the EcoR1 end (terminator side) of the p4H7plasmid linearized with BamH1 & EcoR1. PCR amplification products for *HXT3* and the p4H7 plasmid linearized with EcoR1 and BamH1 were used to yeast as depicted in figure 2. Transformants were selected for their ability to grow on a minimal medium which contained glucose as sole carbon source. The resulting recombined plasmids have the *HXT3* ORF behind the truncated and unregulated *HXT7* promoter leading to overexpression of *HXT3*. All the primers are listed in table 2.

Example 4 Analytical methods

Monitoring of fermentation

CO_2 release was determined by automatic measurement of fermentor weight loss for 20 min each. The CO_2 production rate was automatically calculated by polynomial smoothing of the CO_2 evolved. This method of fermentation monitoring provides high reproducibility. The measure of the total CO_2 evolved was used to check the completion of sugar fermentation. Experiments were done at least in duplicate, representative results are shown.

Monitoring of glucose and fructose consommation

During fermentation, medium is taken at least two times a day, centrifuged to remove cells and supernatant is stored at –20°C before using for glucose and fructose measurement by HPLC.

Example 5 Sequence analysis of *HXT3* genes of the Fermichamp strain

HXT3 gene was amplified by PCR using primers *HXT3*P2 and *HXT3*P1 (primers are shown in table 2.). After purification, PCR products were sequenced and the results are shown in table 3. The promoter region of the *HXT3* gene (nucleotides -900 to 1) displays only 6 mutations while the coding region

(nucleotides 1 to 1700) harbours 38 mutations. Ten of these mutations lead to amino acid changes in the protein sequence when compared with the sequence of the glucophilic wild type strain S288C. Most of these changes are clustered in a region of the protein that includes one membrane spanning domain and an external loop (Figure 1). Most of the changes are conservative substitutions. The *HXT3* gene from strain V5 appeared to be identical to that of S288C.

Table 3. Fructophilic mutations in the *HXT3* gene from Fermichamp ® in comparison with S288C

Promoter (-900 – 1)	ORF (1 – 1700)	Amino acids (1 – 567)
-859 C → T	500 T → C	
-602 A → T	598 A → G	200 **Thr → Ala**
-439 T → deletion	625 A → G	209 **Ile → Val**
-282 A → T	972 G → A	324 **Met → Ile**
-278 T → C	1110 T → C	
-88 C → T	1123 T → C	
	1131 T → C	
	1134 C → T	
	1137 T → C	
	1146 A → C	
	1162 T → A	388 **Leu → Met**
	1164 A → G	388
	1166 A → G	389 **Tyr → Trp**
	1167 T → G	389
	1173 C → T	
	1174 A → G	392 **Ile → Val**
	1176 T → C	392
	1197 A → T	
	1200 T → C	
	1203 C → T	
	1215 T → A	

Promoter (-900 – 1)	ORF (1 – 1700)	Amino acids (1 – 567)
	1221 C → T	
	1225 C → T	
	1240 G → C	414 **Glu → Gln**
	1243 G → A	415 **Gly → Asn**
	1244 G → A	415
	1245 T → C	415
	1248 T → C	
	1251 T → C	
	1311 T → C	
	1317 C → T	
	1320 T → C	
	1326 T → C	
	1341 T → C	
	1445 A → G	449 **Ile → Val**
	1407 T → C	
	1411 T → A	471 **Leu → Ile**
	1413 G → C	471

Example 6: Expression of HXT3 integrated in V5HXT1-7Δstrain

The *HXT3* genes originating either from V5 or Fermichamp were introduced into the V5 HXT1-7Δ strain by integration as indicated in material and methods. Details of the positions of integration at the *HTX3* loci are given in figure 2. After yeast transformation with the PCR products containing the *HXT3* gene, the transformants were directly selected on a medium containing only glucose as carbon source.

The V5 HXT1-7Δ strains containing the integrated *HXT3* gene orginating from V5 or from Fermichamp were obtained and termed V5 HXT1-7Δ*HXT3* (V5) and V5 HXT1-7Δ*HXT3* (Fermichamp) respectively.

The resulting strains were examined for their fermentation properties, fermentation rate and glucose/fructose utilisation. The V5 HXT1-7Δ*HXT3* (V5) and V5

HXT1-7Δ*HXT3* (Fermichamp) displayed different profiles of sugar utilisation (figure 3a, b). The relative rate of fructose and glucose utilisation differ and the strain expressing the *HXT3* gene from Fermichamp displays a higher capacity to use fructose. The ratio glucose/fructose is maintained at higher levels with the strain expressing the *HXT3* gene from Fermichamp.

Comparison of the evolution of the ratios during the fermentation shows that the strain expressing the *HXT3* gene from Fermichamp exhibits a profile similar to the Fermichamp strain while the one expressing the *HXT3* gene from V5 displays a "standard" glucose/fructose profile, similar to Fermivin (Figures 3d, e). The Fermichamp fructose utilisation capacity is therefore triggered in the V5 HXT1-7Δ strain by expression of the Fermichamp *HXT3* gene.

The fermentation rate profiles are also signficantly influenced by the *HXT3* carrier expressed. Although no differences are observed in the first part of the fermentation, a higher fermentation rate is obtained at the end of the fermentation when the gene from Fermichamp is expressed (figure 3c). Consistently, the fermentation time is reduced in recombinants carrying this gene. The better fermentation rate at the end of the fermentation is in agreement with a better capacity to use fructose at the end (which is the main sugar present) and to a minor glucose/fructose disequilibrium in this late phase.

Example 7: Effect of HXT3 gene overexpression

The *HXT3* genes from Fermichamp and from V5 were over expressed in V5 HXT1-7Δ. The *HXT3* gene from Fermichamp or V5 was introduced on a muticopy plasmid, which allows an unregulated and high expression of the corresponding gene (see material and methods).

As shown in figure 4, the overexpression of the *HXT3* genes does not significantly modify the fructose/glucose utilisation of the strain compared to the integrated, single copy, strain. A slight enhancement of the fructose utilisation improvement is however observed.

The overexpression of the *HXT3* genes from V5 and Fermichamp triggers very different effects on the fermentation rate on a MS300 medium containing glucose and frucose (50/50). Overexpression of the *HXT3* gene from V5 has only a little effect on the fermentation rate compared to the integrated single copy (figure 5). Overexpression of the *HXT3* gene from Fermichamp triggers a strong improvement of the fermentation rate and an important reduction of the fermentation time. The

fermentation rate obtained by overexpression of the *HXT3* gene from Fermichamp is much higher than that obtained with the gene from V5.

In order to investigate whether the effect of the overexpression on the fermentation rate was due to a better utilisation of fructose or not, we have examined the fermentation capacity of the *HXT3* overexpressing strains in a MS medium containing only glucose or only fructose. As shown figure 6a, b , the overexpression of *HXT3* from Fermichamp triggers a much higher fermentation rate than the overexpression of the gene from V5, independently of the sugar fermented. The strong improvement of the fermentation capacity compared to the strain expressing the V5 gene, is observed with glucose as well as with fructose. The differences in fermentation capacity triggered by overexpression of the two genes is therefore independent of their fructose transport capacity.

Single copy expression of the *HXT3* genes (in the integrated strains) does not lead to the same picture on pure sugar fermentations (figure 7 a, b). The *HXT3* gene from Fermichamp gives a significant improvement of the fermentation end when only fructose is in the medium. Only a slight difference in fermentation profile is observed between the two genes when glucose is the sugar fermented. This indicate that when the expression of the *HXT3* gene is low, the difference in fermentation rate is mainly due to the capacity to transport fructose

CLAIMS

1) Isolated nucleic acid encoding a functional *HXT3* hexose transporter or fragments thereof with an improved capacity to transport fructose.

2) Isolated nucleic acid according to claim 1 comprising at least one mutation at a position selected from the list consisting of Thr 200, Ile 209, Met 324, Leu 388, Tyr 389, Ile 392, Glu 414, Gly 415, Ile 449, Leu 471.

3) Isolated nucleic acid according to claim 2 comprising at least one mutation at a position selected from the list consisting of Thr 200 Ala, Ile 209 Val, Met 324 Ile, Leu 388Met, Tyr 389 Trp, Ile 392 Val, Glu 414 Gln, Gly 415 Asn, Ile 449 Val, Leu 471 Ile.

4) Isolated nucleic acid according to claim 2 comprising a mutation at or around position Glu 414 or Gly 415.

5) Isolated nucleic acid according to claim 4 comprising a Glu 414 Gln and/or a Gly 415 Asn mutation.

6) Isolated hexose transporter encoded by a nucleic acid according to claims 1 to 4.

7) Recombinant yeast cell transformed with a nucleic acid according to claims 1 to 4

8) Process for obtaining a yeast cell with improved fructophilic properties wherein a recombinant yeast cell comprising a gene encoding an *HXT3* transporter has been altered in such a way that the HXT3 transporter has an improved capacity to transport fructose, comprising the steps of:
- mutating the *HXT3* gene
- selecting the yeast cell with improved fructophilic properties.

ABSTRACT

The present invention relates to yeast strains with improved fructose fermentation capacity, in particular to strains transformed with a gene encoding an improved hexose transporter gene, more in particular a mutated *HXT3* gene. The mutations that individually or in concert attribute to the fructophilic phenotype of a yeast strain are Thr 200 Ala, Ile 209 Val, Met 324 Ile, Leu 388 Met, Tyr 389 Trp, Ile 392 Val, Glu 414 Gln, Gly 415 Asn, Ile 449 Val, Leu 471 Ile, wherein the first three *characters* indicate the amino acid of the wild type *HXT3* protein, the three digits represent the position of the mutation in the protein (Start Met = amino acid number 1) and the last three characters represent the amino acid of the mutated *HXT3* protein according to the invention.

Figure 1 Localisation of mutations in Fermichamp HXT3

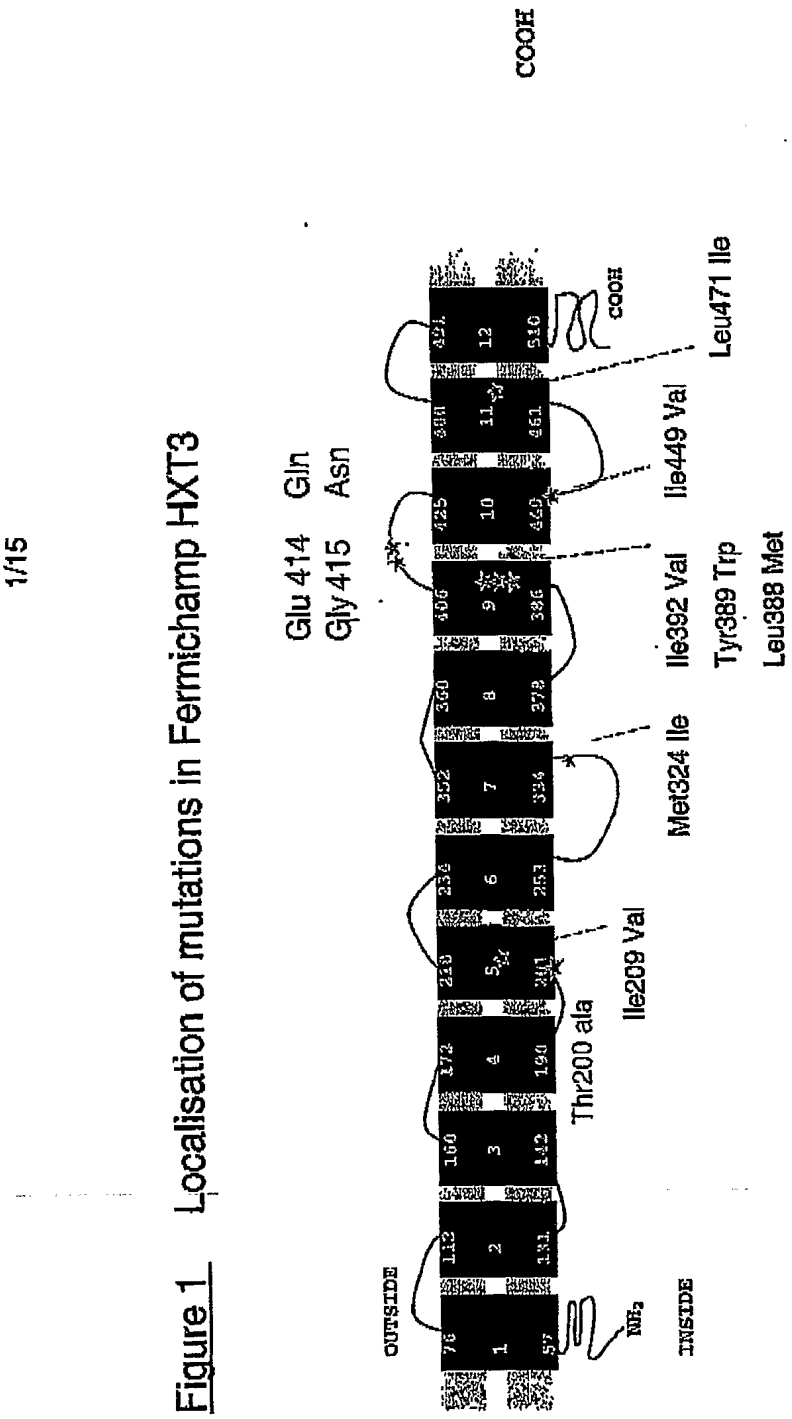

Figure 2A Contruction of V5 strains with integrated *HXT3* genes

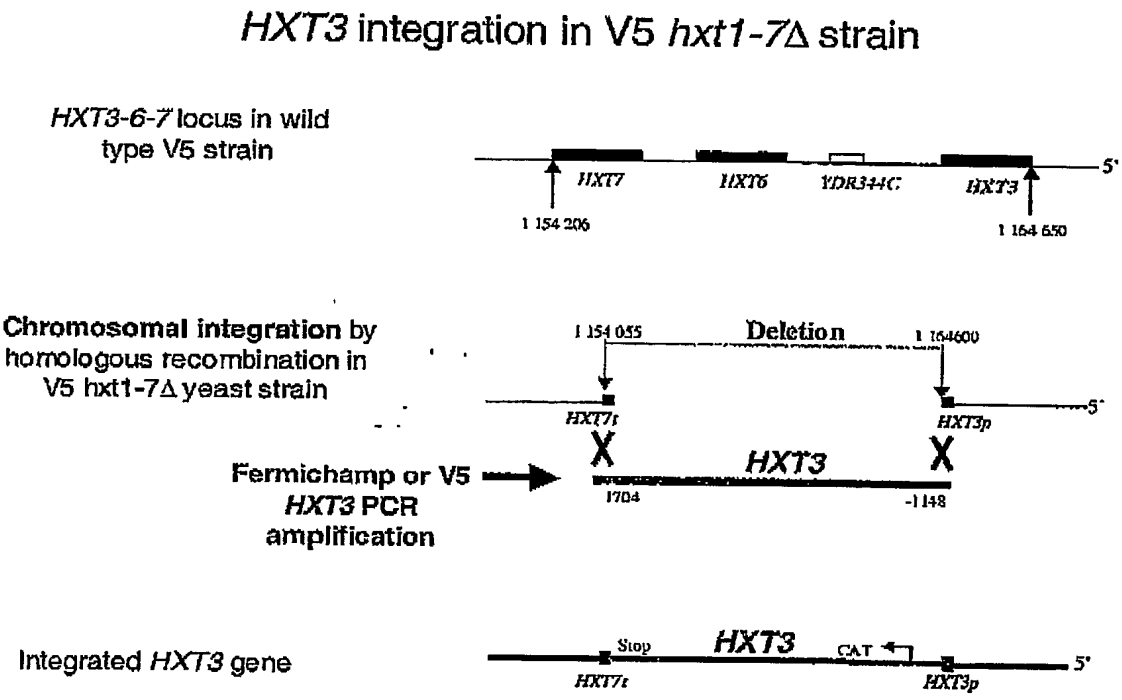

FIGURE 2B : *HXT3* ORF cloning in multicopy plasmid p4H7

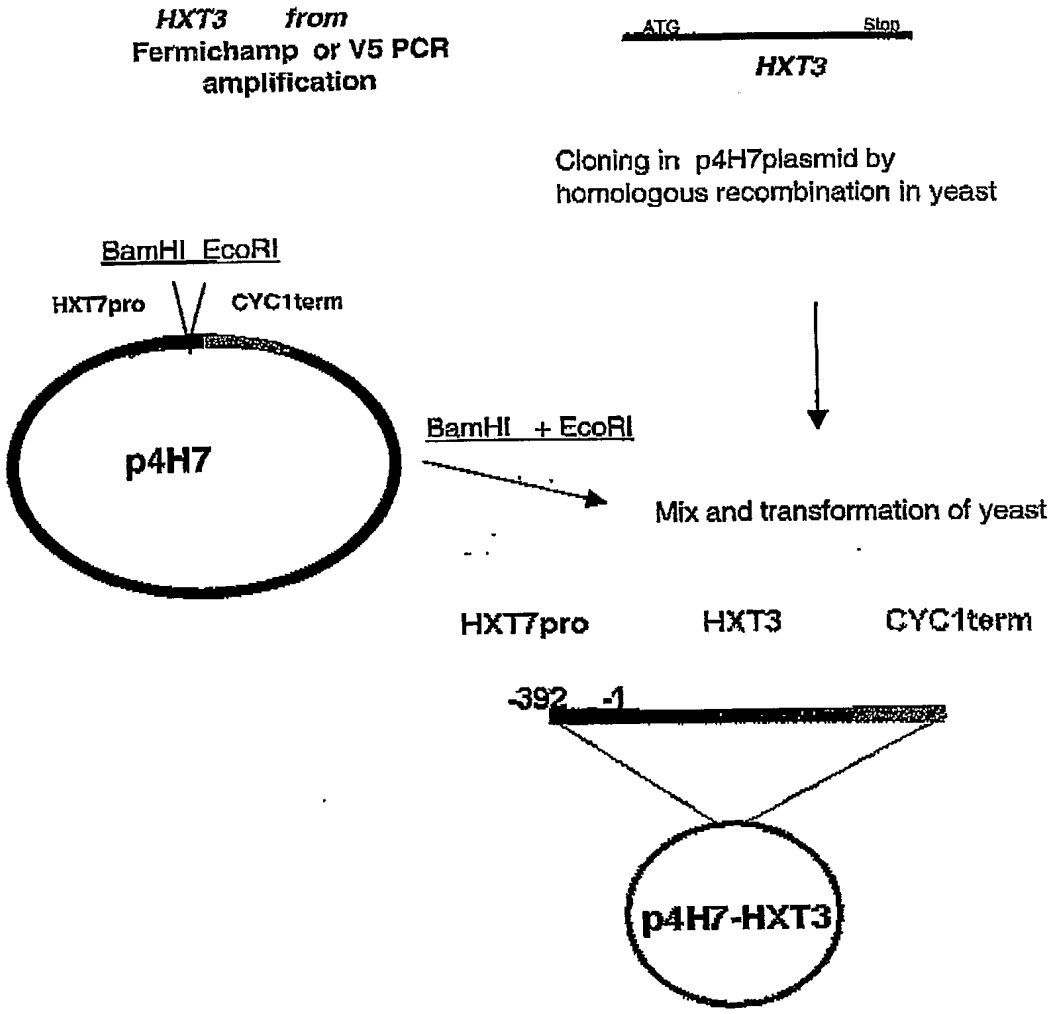

Figure 3A glucose and fructose utilisation by *HXT3* (**V5** or **Fmp**) single copy gene expression

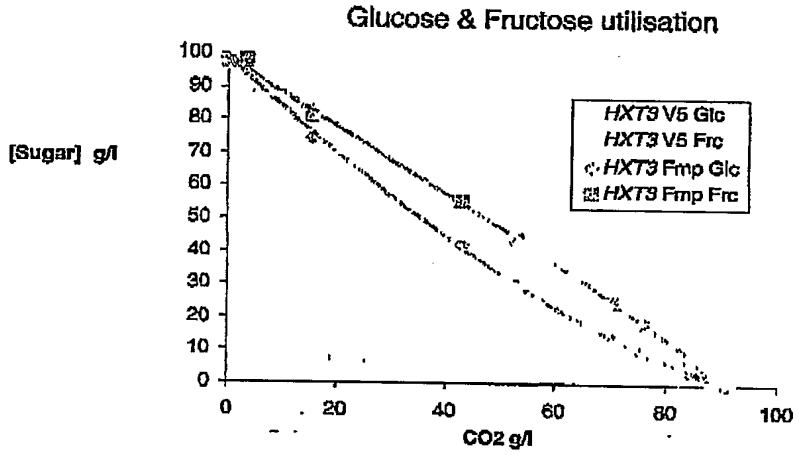

Figure 3B : glucose/fructose ratio of *HXT3* (**V5** or **Fmp**) single copy gene expression

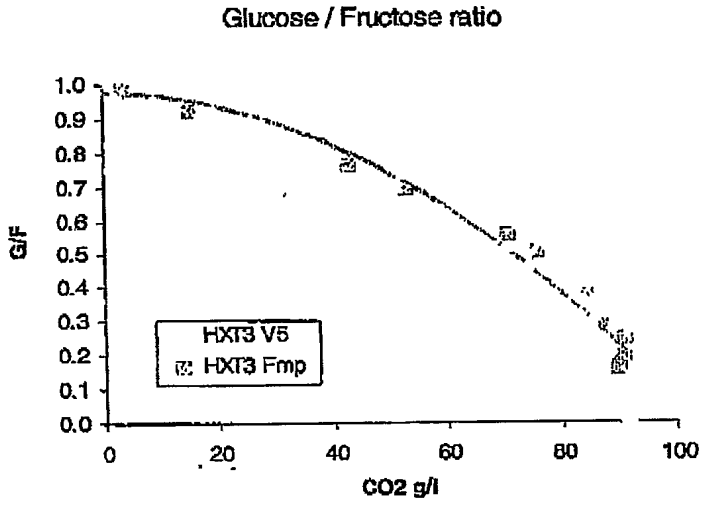

Figure 3C : fermentation rate of *HXT3* (**V5** or **Fmp**) single copy gene expression

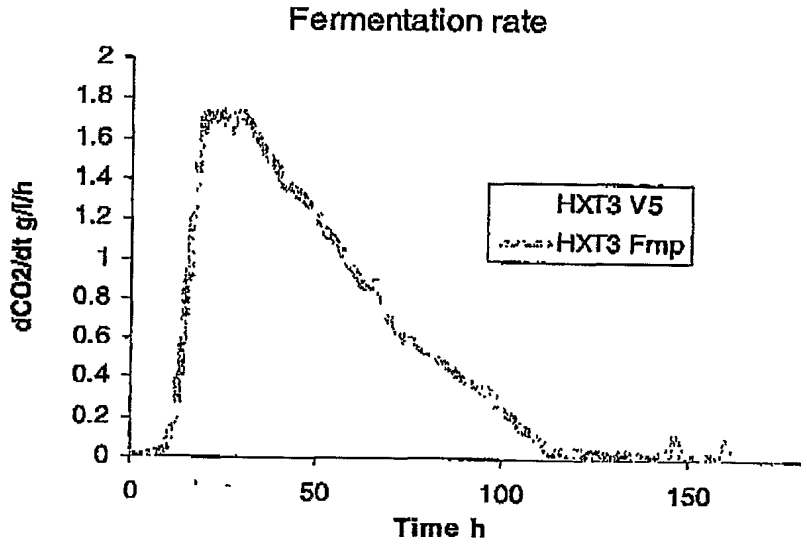

Figure 3D

Comparison of Glucose / Fructose ratio between Fermichamp &

HXT3 (or *Fmp*) single copy gene expression

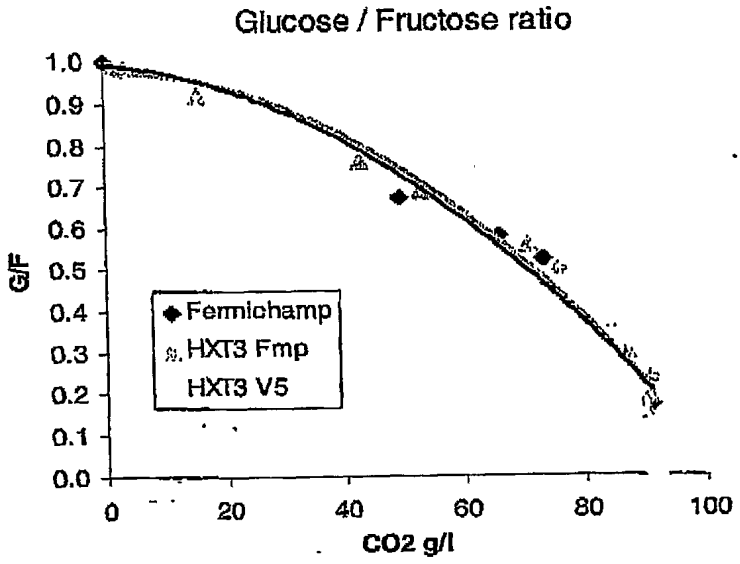

Figure 3 : Comparison of Glucose / Fructose ratio between Fermichamp, Fermivin & *HXT3* single copy gene expression

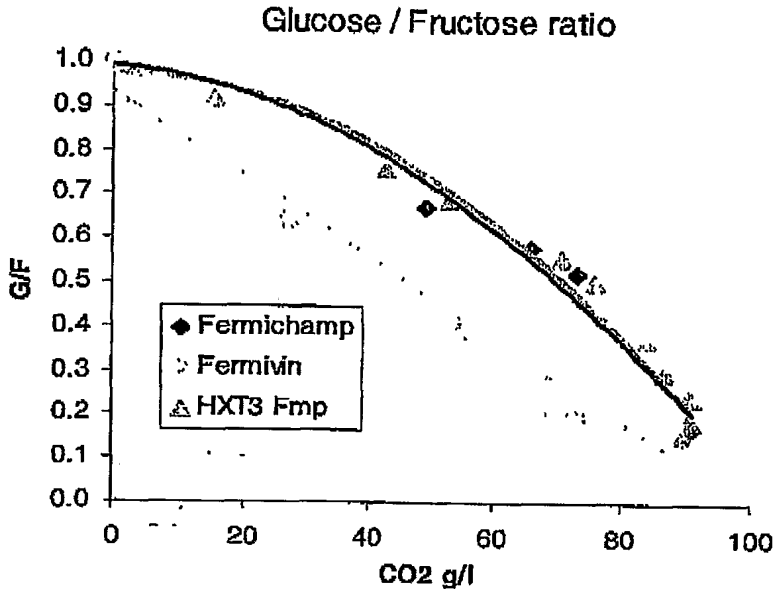

Figure 4A : glucose and fructose utilisation by multicopy overexpression of *HXT3* (V5 or Fmp)

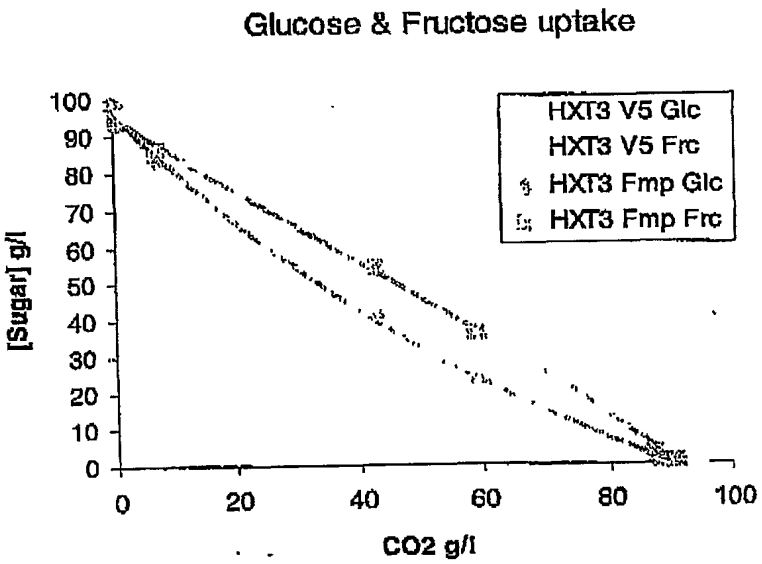

Figure 4B : glucose/fructose ratio by multicopy overexpression of *HXT3* (V5 or Fmp)

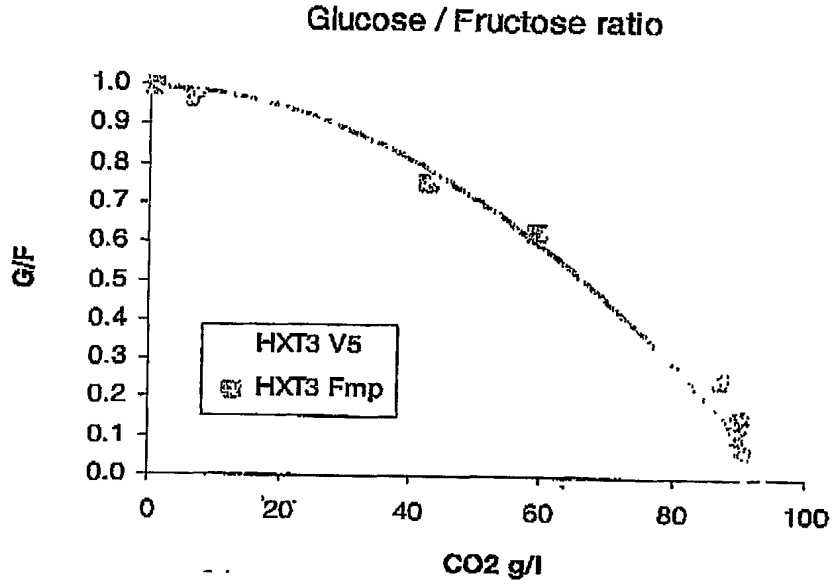

Figure 5 : Multicopy overexpression of *HXT3* (V5 or Fmp) on Glucose + Fructose (50/50) must (200g/l)

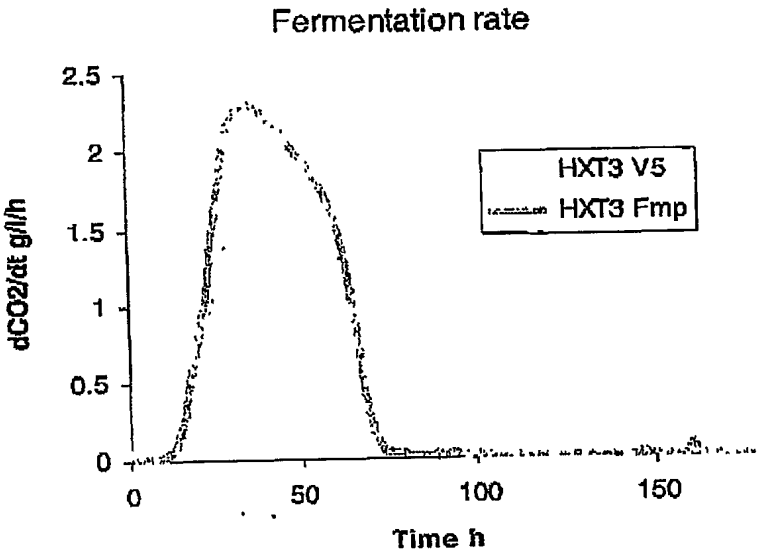

Figure 6A : Multicopy overexpression of *HXT3* (V5 or Fmp) on pure Fructose must (200g/l)

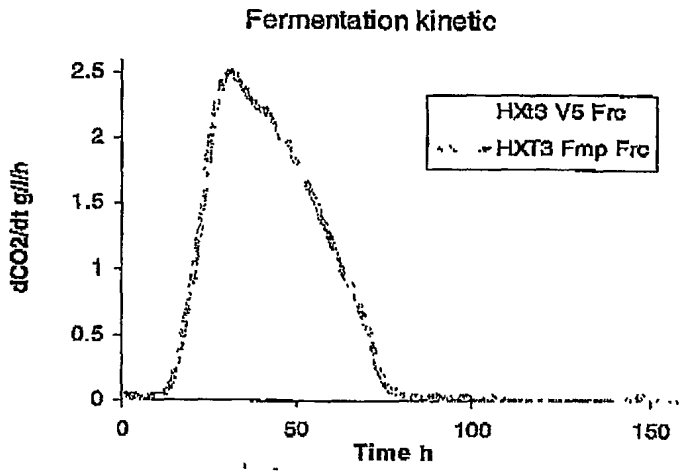

Figure 6B : Multicopy overexpression of *HXT3* (V5 or Fmp) on pure Glucose must (200g/l)

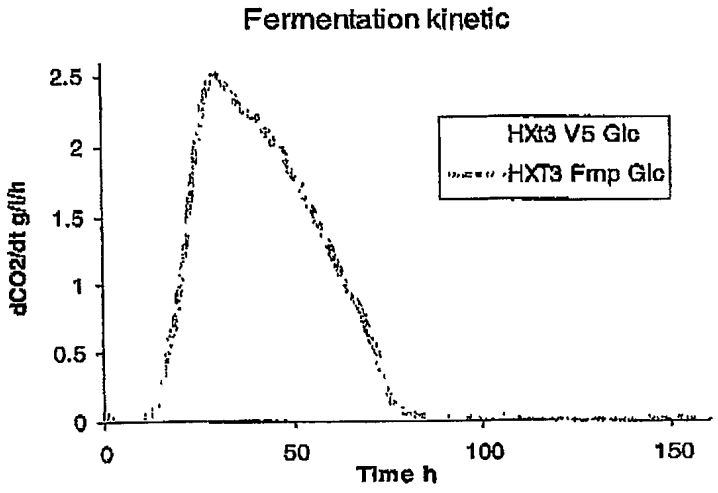

Figure 7A : Single copy expression of *HXT3* (V5 or Fmp) on pure Fructose must (200g/l)

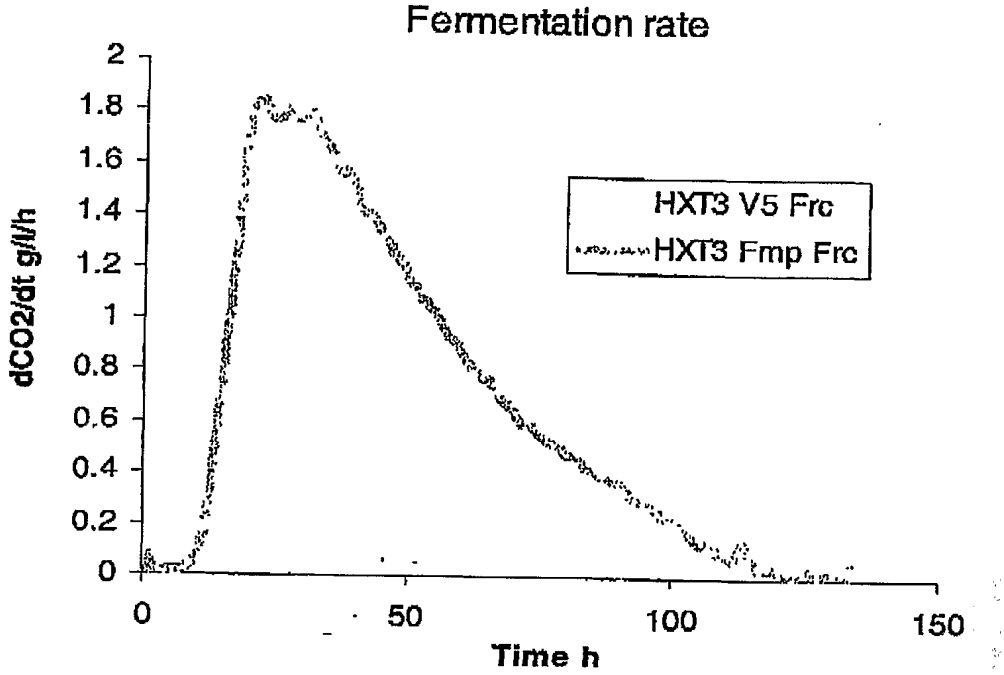

Figure 7B : Single copy expression of *HXT3* (V5 or Fmp) on pure Glucose must (200g/l)